

## Research paper

# Usefulness of IL-21, IL-7, and IL-15 conditioned media for expansion of antigen-specific CD8<sup>+</sup> T cells from healthy donor-PBMCs suitable for immunotherapy

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## ABSTRACT

Clonal anergy and depletion of antigen-specific CD8<sup>+</sup> T cells are characteristics of immunosuppressed patients such as cancer and post-transplant patients. This has promoted translational research on the adoptive transfer of T cells to restore the antigen-specific cellular immunity in these patients. In the present work, we compared the capability of PBMCs and two types of mature monocyte-derived DCs (moDCs) to prime and to expand *ex-vivo* antigen-specific CD8<sup>+</sup> T cells using culture conditioned media supplemented with IL-7, IL-15, and IL-21. The data obtained suggest that protocols involving moDCs are as efficient as PBMCs-based cultures in expanding antigen-specific CD8<sup>+</sup> T cell to ELA and CMV model epitopes. These three gamma common chain cytokines promote the expansion of naïve-like and central memory CD8<sup>+</sup> T cells in PBMCs-based cultures and the expansion of effector memory T cells when moDCs were used. Our results provide new insights into the use of media supplemented with IL-7, IL-15, and IL-21 for the *in-vitro* expansion of early-differentiated antigen-specific CD8<sup>+</sup> T cells for immunotherapy purposes.

## 1. Introduction

The immune system is a complex set of cells, organized into tissues and molecules that protect the host from infection by pathogens, abnormal cell growth, and the development of autoimmune diseases. Within this system, dendritic cells (DCs) play a central role in the communication between the innate and adaptive immune responses. DCs reside in different tissues where they act as sentinels capturing and processing antigens that are subsequently presented in the context of MHC class I and II molecules to T lymphocytes inducing their activation, differentiation, and expansion, a key step for the acquisition of antigen-specific cellular immunity [1–3].

Cancer is one of the non-communicable diseases causing the highest morbidity and mortality in the world and generating a high economic

impact on health systems. The increase in the incidence of both solid and hematological tumors poses a serious global public health problem. In addition, there is a lack of evidence to allow early diagnosis of most tumor diseases and, therefore, the prognosis in advanced stages is usually quite poor. In these stages, conventional cancer treatments such as surgery, chemotherapy and radiotherapy do not have the necessary therapeutic scope to control the tumor. In turn, infections caused by latent and lytic viruses like Cytomegalovirus (CMV), Epstein-Barr virus (EBV), and Adenovirus (Adv) are the main cause of morbidity and mortality in post-transplant immunosuppressed patients [4,5]. To address this, in the last thirty years, research has focused on two approaches: (i) the adoptive transfer of antigen-specific T cells after the *in-vitro* expansion of autologous T cells from the circulating lymphocyte pool (either PBMCs or enriched CD8<sup>+</sup> T-cell preparations) using

**Abbreviations:** α1DCs, alpha-polarized type 1 dendritic cells; Adv, adenovirus; ACT, adoptive cell transfer; APCs, Antigen-presenting cells; CMV, cytomegalovirus; CTL, cytotoxic T cells; DCs, Dendritic cells; EBV, Epstein-Barr virus; iDCs, immature dendritic cells; IL, Interleukin; MPLA, Monophosphoryl Lipid A; moDCs, monocyte-derived dendritic cells; PBMCs, Peripheral Blood Mononuclear Cells; stDCs, standard dendritic cells; T<sub>N</sub>, naïve T cells; T<sub>SCM</sub>, Stem cell-like memory T cells; T<sub>CM</sub>, Central memory T cells; T<sub>EM</sub>, Effector memory T cells; TLR, Toll-like receptor.

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professional antigen-presenting cells (natural or artificial DCs) and (ii) the use of DCs-based vaccines that induce activation and *in-vivo* expansion of antigen-specific T cells.

Even though DCs-based vaccines have led to the induction of measurable antigen-specific CTL response in vaccinated individuals, the magnitude of T cell induction is limited and its impact on tumor progression is not as strong as needed. Adoptive cell transfer (ACT) of antigen-specific CTLs is a promising approach to increase anti-tumor and anti-viral immunological responses in cancer and post-transplant patients [6–8]. The evidence indicates that such therapy is feasible, safe and well-tolerated and offers an alternative to conventional treatments. Although some clinical trials have shown that ACT induces tumor regression in cancer patients or the control of viral agents in high-risk transplant recipients, there are still many questions to be answered to establish a standard protocol that can be widely applied in the clinical settings.

The protocols widely used for over thirty years have the ability to expand antigen-specific CD8<sup>+</sup> T cells with a highly differentiated phenotype (CD27<sup>low</sup>CD28<sup>+</sup>). In 2011, Gattinoni *et al.* described a subtype of T cells called stem cell-like memory T cells (T<sub>SCM</sub>) identifiable by the expression of the markers CD45RA<sup>+</sup>CCR7<sup>+</sup>CD27<sup>+</sup>CD28<sup>+</sup>CD62L<sup>+</sup> typical of naïve T cells but also expressing CD95 and the beta receptor of IL-2 in a similar way to conventional memory T cells [9]. One of the greatest challenges of ACT is to obtain enough numbers of early-differentiated T cell populations (T<sub>SCM</sub> and T<sub>CM</sub>) to infuse into the patient. Both populations (T<sub>SCM</sub> and T<sub>CM</sub>) circulate between the lymphoid nodes and have a high capacity for expansion after activation; in contrast, more differentiated populations such as effector or memory effector cells migrate into the tissues and respond immediately to the antigen, but do not survive over time because of their limited capacity for self-renewal [10–13]. To expand antigen-specific CD8<sup>+</sup> T cells suitable for ACT, distinct *ex-vivo* culture conditions using a variety of APCs, cytokines, and the starting cells are being evaluated. Studies to date have found that the homeostatic control of T<sub>SCM</sub> depends on the cytokines IL-7, IL-15, and IL-21 of the common gamma chain family [14–16], therefore, their use in the preparedness of T cells for ACT is very attractive.

Here, we have deepened the study of culture conditions for the *in-vitro* expansion of CD8<sup>+</sup> T cells with early-differentiated memory phenotype and low levels of exhaustion markers available for ACT. To expand antigen-specific CD8<sup>+</sup> T cells, we compared a 10 days long culture system based on CD8<sup>+</sup> T cells activated by either standard DCs (stDCs) or  $\alpha$ -type 1 polarized DCs ( $\alpha$ 1DCs) pulsed with peptide, Vs. PBMCs pulsed with a single round of peptide stimulation. The culture media was supplemented with IL-21 during the first 72 h of priming and later with three doses of IL-7 and IL-15 or IL-2. Using MHC class I/peptide fluorescent multimers, we have analyzed in detail the influence of the starting cells (enriched-CD8<sup>+</sup> T cells Vs. PBMCs), the usefulness of moDCs as APCs and cytokines for the *in-vitro* expansion of CD8<sup>+</sup> T cells from healthy donors with naïve or antigen-experienced phenotype specific for two model antigens: Melan-A/Mart-1<sub>26-35A27L</sub> and CMV pp65<sub>495-503</sub>.

## 2. Materials and methods

### 2.1. Reagents

Fluorescent-labelled antibodies specific for CD80, CD83, CD54, CD8, CD45RA, CCR7, CXCR3, CD69, 41BB, PD-1, CTLA-4 and KLRG-1 were obtained from BioLegend. Biotinylated HLA-A\*02:01 tetramers were synthesized at the Lawrence Stern Laboratory, University of Massachusetts Medical school by CP, and then were labeled with streptavidin-Phycoerythrin (PE) (Invitrogen) at a 4:1 M ratio before use. PE-labelled HLA-A2/peptide tetramers were also purchased from MHC Tetramer Core, Baylor College of Medicine, Houston, USA. Cytokine secretion (IL-12p70) was measured in the culture supernatants using the human Inflammatory CBA kit (BD Biosciences). The Melan-A/Mart-1<sub>26-35A27L</sub>

(ELAGIGILTV) and CMV pp65<sub>495-503</sub> (NLVPMVATV) peptides were generated through solid-phase peptide synthesis (21st Century Biochemicals, Inc, Marlborough, Massachusetts, USA). Lyophilized peptides were reconstituted first in 1% DMSO (v/v) and then we completed the volume with water for a final concentration of 1 mg/mL. The samples were acquired using the FACS Aria III System at the Universidad Nacional de Colombia - School of Medicine. The flow cytometry data was exported in FCS format v3 and analyzed using FlowJo software (TreeStar Inc.). The graphics were generated using Prism v8 software (Graph Pad).

### 2.2. Donors and cells

Buffy coat from 10 healthy donors (5 male and 5 female between 18 and 45 years old without oncologic, autoimmune or recent infectious diseases) was obtained from whole blood samples that were collected by the blood bank of the District Institute of Science, Biotechnology and Innovation in Health (IDCBIS) and who generously donated them for the purposes of this study. The units were subjected to HLA-A2 typing by flow cytometry and then HLA-A\*02:01 haplotype was confirmed by high-resolution HLA-A typing (Exons 2 and 3) by Histogenetics LLC. PBMCs were purified using density gradient Ficoll and cryo-preserved in RPMI-1640 + 40% fetal calf serum (FCS) + 10% dimethyl sulfoxide (DMSO). The frequencies of *ex-vivo* Melan-A/Mart-1<sub>26-35A27L</sub> and CMV pp65<sub>495-503</sub> multimer positive CD8<sup>+</sup> T cells were determined for each donor. Each experiment was performed with the cells from each donor and some comparative experiments was performed with the same donors' cells. We specify this in each chart.

### 2.3. CTL generation – Peptide stimulation of PBMCs

#### 2.3.1. PBMCs stimulation

4 × 10<sup>6</sup> fresh/frozen PBMCs were resuspended in CTL media (AIM-V with 5% human AB serum (v/v)) alone or pulsed with peptide at a concentration of 20 µg/mL and supplemented with cytokines (as outlined below). Each treatment (Mart1 or CMVpp65) was evaluated separately. Cells were plated out in a 96-well round-bottomed plate (5 × 10<sup>5</sup>/well). Media and cytokines were replenished on days 3, 5 and 7. On day 10 CTLs were harvested and used for phenotypic studies.

#### 2.3.2. Cytokines for promoting CTL activation and expansion

We compared three conditions; (i) no cytokines, (ii) IL-21 (30 ng/mL) at day 0 + IL-7 and IL-15 (5 ng/mL) on days 3 and 5 and IL-7 and IL-15 (10 ng/mL) on day 7, and (iii) IL-21 (30 ng/mL) at day 0 + IL-2 (100 U/mL) on days 3, 5 and 7. All cytokines were purchased from CellGenix, Portsmouth, New Hampshire, USA.

### 2.4. CTL generation using peptide-pulsed moDCs

#### 2.4.1. Generation of dendritic cells

Mature-monocyte-derived dendritic cells (moDCs) were generated from frozen PBMCs, as described previously [17]. Briefly, autologous dendritic cells were prepared from the adherent monocyte fraction by culture for 2 days in DC media (AIM-V with 1% human AB serum (v/v)) with IL-4 (40 U/mL) and GM-CSF (800 U/mL) and for another 24 h with IL-4 (40 U/mL) and GM-CSF (1600 U/mL). To continue our research project, iDCs were matured using two different cocktails: (i) the *standard cocktail*, containing IL-6 (1000 U/mL), IL-1 $\beta$  (10 ng/mL), TNF $\alpha$  (10 ng/mL) (all from CellGenix) and PGE2 (1 µg/mL, Sigma-Aldrich) for 16–24 h; or the  *$\alpha$ -type 1 polarized cocktail*, containing IL-6 (1000 U/mL), IL-1 $\beta$  (10 ng/mL), TNF $\alpha$  (10 ng/mL) (all from CellGenix), IFN $\gamma$  (100 U/mL, R&D Systems), IFN $\alpha$  (1000 U/mL, Intron-A; Schering Plough Corp., Kenilworth, NJ) and Poly I:C (20 µg/mL, Sigma-Aldrich) for 16–24 h. A third maturation cocktail, the *LPS cocktail* containing IFN $\gamma$  (100 U/mL, R&D Systems) and LPS (10 ng/mL) was used to standardize a four-day maturation protocol instead the classic seven-day maturation protocol.

LPS-DCs were not used in all the experiments settings. The mature phenotype was evaluated by flow cytometry quantifying the expression levels of CD80, CD83 and CD54.

#### 2.4.2. CD8+ T-cell enrichment

CD8+ T lymphocytes were purified from PBMCs by negative selection by depletion of non-target magnetically labeled cells (Miltenyi Biotec, Bergisch Gladbach, Germany). Naïve CD8+ T ( $T_N$ ) lymphocytes were purified from PBMCs by negative selection using the EasySep™ Human naïve CD8+ T Cell Isolation Kit according to the manufacturer's instructions (StemCell Technologies, Seattle, WA, USA). The purity of enrichment fraction was confirmed by flow cytometry using fluorescent-labeled antibodies specific for CD45RA and CCR7.

#### 2.4.3. CD8 T-cell activation

Once obtained, pulsed or unpulsed moDCs were cocultured with autologous-enriched CD8+ T lymphocytes in a ratio of 4:1 (T cells: moDCs). Cells were plated out in a 48-well plate ( $5 \times 10^5$  enriched T cells per well in 500  $\mu$ L of CTL media). Media and cytokines were replenished on days 3, 5 and 7. On day 10 CTLs were harvested and used for phenotypic studies.

#### 2.4.4. Cytokines for promoting CTL activation and expansion

IL-21 (30 ng/mL) was added to the cell mixture on day 0. IL-7 and IL-15 were used at a concentration of 5 ng/mL on days 3 and 5, and at 10 ng/mL on day 7. All cytokines were purchased from CellGenix, Portsmouth, New Hampshire, USA.

### 2.5. Phenotype and functional analysis

#### 2.5.1. Tetramer and surface antibodies staining

At the end of the expansion cultures, cells were harvested, washed once with phosphate-buffered saline (PBS) (Sigma), pelleted, and stained with specific PE-tetramers. After 1 h at room temperature in the dark cells were surface-stained with monoclonal antibodies to CD8, CD45RA, CCR7, CD69, 41BB, PD-1, CTLA-4 and KLRG-1. After 20 min at 4 °C in the dark, cells were washed twice and analyzed.

#### 2.5.2. Identification of Memory T cell populations

The different memory T cell populations were determined by the surface expression of CD45RA and CCR7 markers:  $T_N$  (CD45RA<sup>+</sup>CCR7<sup>+</sup>),  $T_{CM}$  (CD45RA<sup>+</sup>CCR7<sup>+</sup>),  $T_{EM}$  (CD45RA<sup>-</sup>CCR7<sup>+</sup>) and  $T_{EMRA}$  (CD45RA<sup>+</sup>CCR7<sup>-</sup>).  $T_{SCM}$  was defined by the expression of the additional marker CXCR3 (CD45RA<sup>+</sup>CCR7<sup>+</sup>CXCR3<sup>+</sup>).

#### 2.5.3. Functional analysis of moDCs

To evaluate the functional capacity of moDCs activating T cells, we coculture Melan-A/Mart-1<sub>26-35A27L</sub>-specific CD8+ T cells (kindly provided by Dr. P. Romero, Ludwig Institute for Cancer Research, Lausanne, Switzerland) with both peptide-pulsed and unpulsed  $\alpha$ 1DCs and stDCs. After 48 h CD8+ T cells were harvested and stained with fluorescent-labeled antibodies specific for CD69 and 41BB as activation markers. IL-12p70 production was evaluated by CBA (BD Biosciences) in the supernatant of cell cultures.

### 2.6. Statistical analysis

Results were evaluated using descriptive statistics (means, SDs, and ranges). Since most of the readouts did not present a normal distribution, non-parametric tests were applied. The student's *t* test was performed for comparison of the percentage of expansion of tetramer-specific CD8+ T cells between stimulated and unstimulated groups. Differences between three or more groups were analyzed with One-way ANOVA with Kruskal-Wallis test. Two-way ANOVA was performed for comparison of  $T_N$  and  $T_{SCM}$  between treatment groups. Statistical analyses were done using GraphPad Prism version 8.0 (GraphPad software,

La Jolla, CA). Comparisons determined to be  $p > 0.05$  but  $> 0.01$  were labelled \*,  $p \leq 0.01$  but  $> 0.001$  \*\*,  $p \leq 0.011$  but  $> 0.0001$  \*\*\*, and  $p \leq 0.0001$  \*\*\*\*. ns = not significant.

## 3. Results

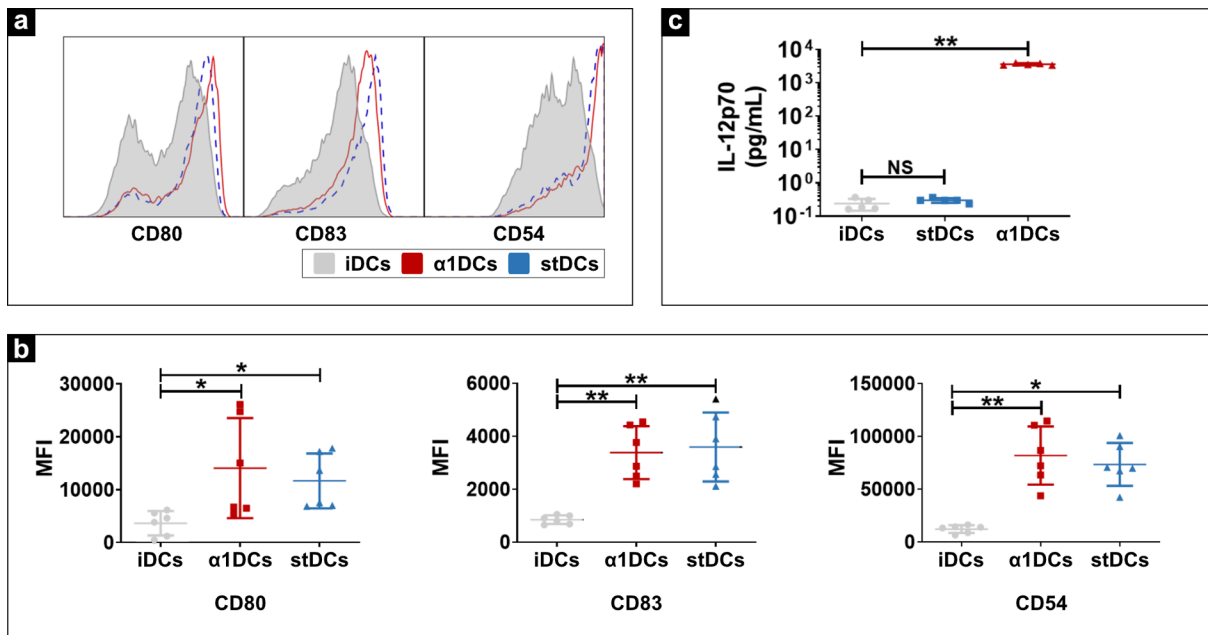
### 3.1. $\alpha$ 1DCs have similar maturation phenotype to stDCs with increased production of IL12p70

Based on the work of Wöfl and Greenberg, we first obtained moDCs through the plastic-adherence method using a four-day long intermediate-term protocol. This moDCs showed an increased expression of CD80, CD83 and CD54 markers reflecting a characteristic mature phenotype ( $n = 6$ ) (Fig. 1a – b; Supplementary Fig S1a). The mature phenotype exhibited by four-day moDCs is similar to moDCs obtained in protocols of two and seven days (based on previous studies of our and other groups). As previously reported, we confirmed the IL12p70 production by  $\alpha$ 1DCs and LPS-DCs but not by stDCs (Fig. 1c; Supplementary Fig S1b). Then we performed a simple but practical assay to evaluate the functional capacity of moDCs. We co-cultured peptide-pulsed or non-pulsed  $\alpha$ 1DCs and stDCs with purified CD8+ T cells clones specific for Melan-A/Mart-1<sub>26-35A27L</sub> and confirmed the proficiency as antigen-presenting cells of both types of DCs by assessing the expression of the surface activation markers CD69 and 41BB on these CD8+ T cells upon activation. (Supplementary Fig S2).

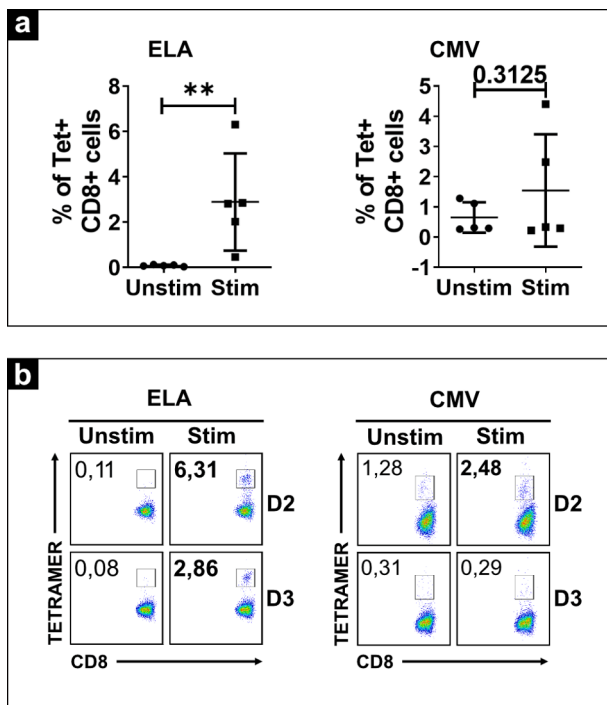
### 3.2. Peptide-stimulated PBMCs and peptide pulsed moDCs as APCs drive strong expansion of both naïve and antigen-experienced CD8+ T cells

Even though mature DCs may be particularly efficient in expanding T cells *in-vitro*, the process to obtain them is complex and wasteful. Some critical factors such the expertise needed to obtain them and the process to standardize the ratio of mDCs:T cells are involved. The above can be avoided by using the cellular subfraction of PBMCs that contains a variety of APCs such natural DCs, macrophages and B cells. That is the reason why *in-vitro* single-round or repeated stimulation of PBMCs with antigenic peptides remains a method of choice for the amplification of antigen-specific T cells. We first carried out a single round of PBMCs peptide stimulation in media supplemented with one dose of IL-21 followed by three doses of IL-7 and IL-15. A significant expansion of antigen-specific CD8+ T cells was attained after 10 days of culture. All donors showed a great expansion ( $0.086 \pm 0.036\%$  and  $2.894 \pm 2.143\%$ ; unstimulated and stimulated respectively) of T cells specific for the Melan-A/Mart-1<sub>26-35A27L</sub> epitope and 2 of 5 donors showed a significant expansion ( $0.65 \pm 0.50\%$  and  $1.54 \pm 1.86\%$ ; unstimulated and stimulated respectively) for the CMV pp65<sub>495-503</sub> epitope ( $n = 5$ ) (Fig. 2a - b). To corroborate these results, PBMCs were stimulated or not with Melan-A/Mart-1<sub>26-35A27L</sub> peptide, and then expanded without cytokines, or with media supplemented with (i) IL-21 (30 ng/mL) at day 0 + IL-7 and IL-15 (5 ng/mL) on days 3 and 5 and IL-7 and IL-15 (10 ng/mL) on day 7, or (ii) IL-2 (100 U/mL) (Fig. 3a). Cultures supplemented with IL-21 + IL-7 + IL-15 or IL-21 + IL-2 showed a significant expansion of antigen-specific CD8+ T cells ( $2.64 \pm 1.73\%$  and  $8.61 \pm 3.25\%$  respectively). Cultures that were stimulated in the absence of cytokines did not expand ( $0.059 \pm 0.016$ ). No differences between both cytokine conditions were observed (Fig. 3b-c). These results suggest that both high variabilities of responsiveness among donors and different antigenic requirements between non-primed naïve T cells and memory T cells (e.g., those specific for Melan-A/Mart-1<sub>26-35A27L</sub> and for CMV pp65<sub>495-503</sub> epitopes respectively) might be limiting factors for ACT.

Melan-A and CMV pp65-specific CD8+ T cells were also stimulated with autologous moDCs loaded with peptide. In exploratory experiments, we found that the expansion of Melan-A-specific CD8+ T cell-driven by LPS-DCs was larger than that achieved in peptide-stimulated PBMCs cultures ( $8.10 \pm 5.86\%$  and  $2.894 \pm 2.143\%$ ; respectively) ( $n = 5$ ) (Supplementary Fig. S3a). Taking into account the experience of



**Fig. 1.** Phenotypic and functional characteristics of four-day moDCs. a) Histograms representing the expression of maturation markers CD80, CD83, and CD54 on immature dendritic cells (gray histogram), α1DCs (red line) and stDCs (blue line). Results of experiments presented are representative of four performed; b) scatter plot summarizing expression levels of maturation markers CD80, CD83 and CD54 between iDCs, α1DCs and stDCs (n = 6); c) scatter plot shown the supernatant quantification of IL-12p70 secreted by iDCs, α1DCs and stDCs.

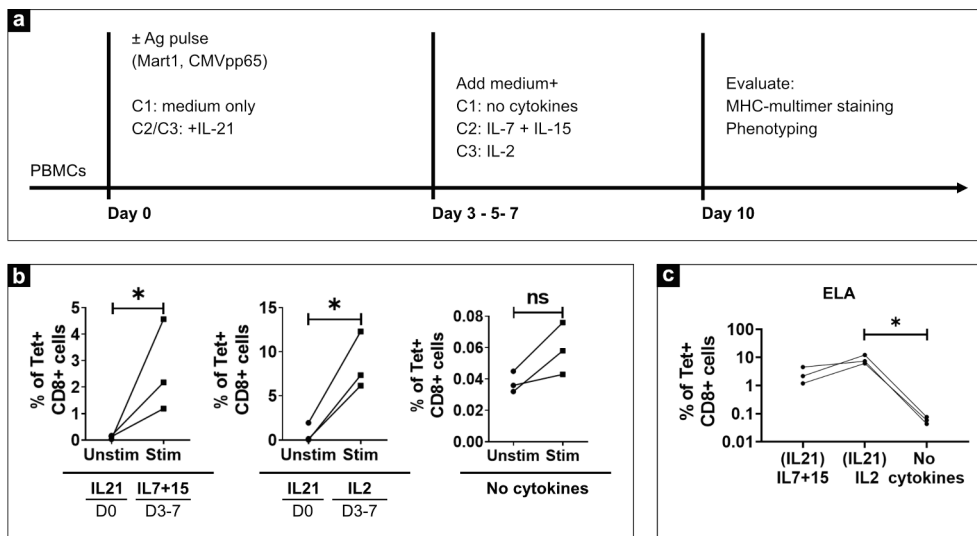


**Fig. 2.** Peptide-stimulated PBMCs drive the expansion of antigen-specific CD8+ T cells. a) Percentage of tetramer-positive CD8+ T cells expanded from PBMCs (donors 2–6) stimulated or unstimulated with Melan-A/Mart-1<sub>26-35A27L</sub> epitope (left panel) and CMV pp65<sub>495-503</sub> epitope (right panel); b) Representative dot plots of tetramer specific CD8+ T cells after 10 days of *in-vitro* stimulation of PBMCs with the corresponding peptide. The number inside the plots represents the percentage of tetramer-specific CD8+ T cells. Results of experiments presented in panel b are representative of five performed (donors 2 – 6).

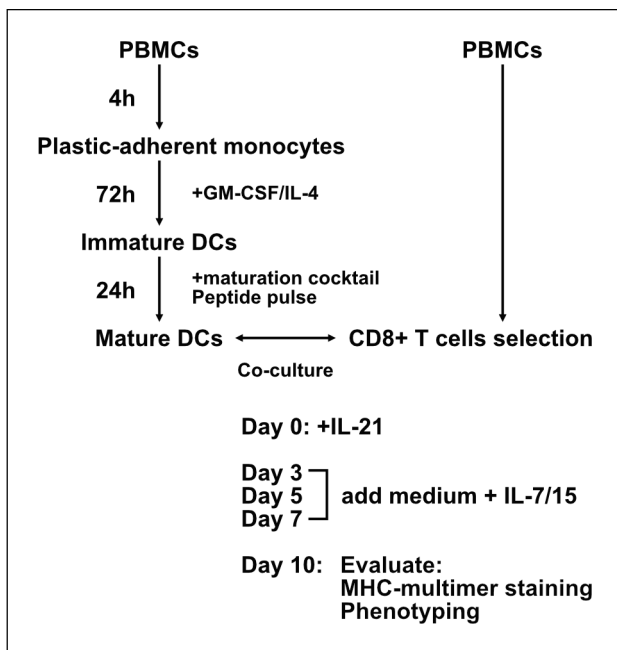
our group in the implementation of dendritic cell-based immunotherapy using α1DCs and stDCs for the treatment of breast cancer patients (Bernal-Estevéz D. et al., manuscript in preparation), we evaluated the ability of these two types of moDCs to induce the expansion of Melan-A/Mart-1<sub>26-35A27L</sub> and CMV pp65<sub>495-503</sub> antigen-specific T cells from enriched-CD8+ T cell samples (Fig. 4). Our results showed that although both types of maturation cocktails favor the expansion of antigen-specific CD8+ T cells, it is observed that α1DCs drive a higher degree of expansion for the Melan-A epitope than stDCs (2.41 ± 0.75% and 1.035 ± 0.38%; α1DCs and stDCs respectively); whereas no difference between both moDCs was found for the CMV epitope (4.81 ± 3.79% and 1.77 ± 0.61%; α1DCs and stDCs respectively) (n = 4) (Fig. 5a – b). No differences between PBMCs and moDCs-based cultures in the overall expansion of T cells for the Melan-A epitope was observed, whereas moDCs were more efficient than PBMCs in fostering the expansion of T cells specific for the CMV pp65 epitope (PBMCs n = 5; moDCs n = 4) (Fig. 6).

### 3.3. IL-21, IL-7, and IL-15 promote the expansion of antigen-specific CD8+ T cells with an early-differentiated memory phenotype

Based on the very low or null numbers of the *ex-vivo* population of CD8+ T cells specific to Melan-A and CMVpp65 between donors, we hypothesize that the potential expansion of antigen-specific CD8+ T cells would come from the naïve repertoire. Considering that the relationship between the T cell differentiation phenotype and the anti-tumor and anti-viral capacity of T cells has become an important issue, we compared the use of the common gamma chain cytokines IL-7, IL-15 and IL-21 that are known as cytokines that support naïve and memory T cell regeneration and proliferation for the antigen-driven expansion of naïve and memory CD8+ T cells versus the effect of IL-2. By examining the differential expression of CD45RA and CCR7, we compared the memory phenotype on Melan-A and CMV-specific CD8+ T cells emerging either from peptide-stimulated PBMCs or from CD8+ T cells cultured with antigen-pulsed moDCs. In a first set of experiments, we found that in cultures of peptide-stimulated PBMCs a single dose of IL-21 followed by relatively low doses of IL-7 in combination with IL-15, promotes the



**Fig. 3. Growth-promoting cytokines enhance the expansion of antigen-specific CD8+ T cells.** a) Experimental outline with PBMCs: PBMCs were pulsed or not with peptide on day 0 and IL-21 was added to conditions 2 (C2) and 3 (C3). After 72 h fresh medium supplemented with IL-7 + 15 was added to C2 and with IL-2 to C3. MHC-multimer staining and phenotype analysis was performed on day 10. b) Percentage of tetramer-positive CD8+ T cells expanded from PBMCs stimulated or unstimulated with Melan-A/Mart-1<sub>26-35A27L</sub> epitope in the presence of IL-21 + IL-7 + 15, IL-21 + IL-2 or without exogenous cytokines; c) Cell expansion of tetramer specific CD8+ T cells after 10 days of *in-vitro* stimulation of PBMCs with Melan-A/Mart-1<sub>26-35A27L</sub> epitope in media supplemented with or without different Th1, proliferative and prosurvival cytokines. *n* = 3, (donors 1–3).



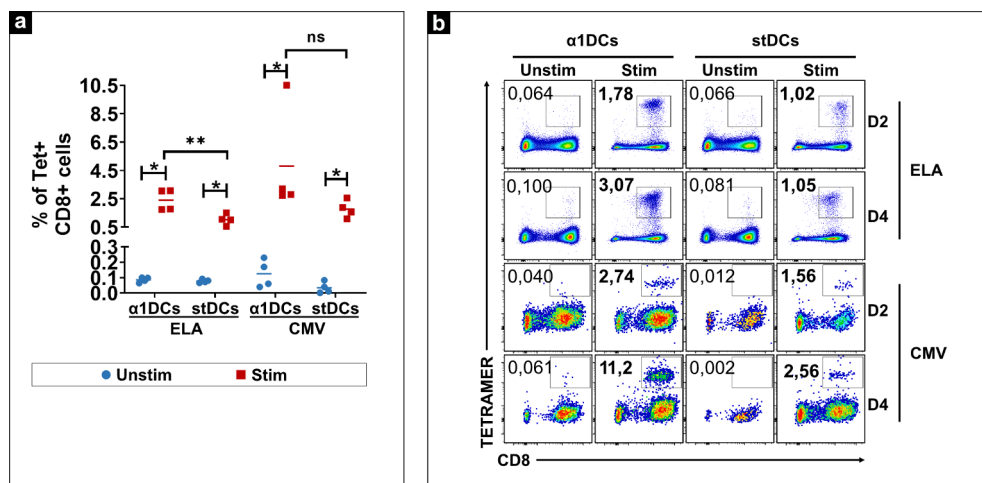
**Fig. 4. Workflow of rapid expansion of antigen-reactive CTLs using moDCs.** Start with monocyte isolation and DCs derivation on day –4 of co-culture, followed by CD8+ T cell isolation on day –1 of co-culture. Initiation of co-culture of peptide-pulsed or unpulsed DCs with CD8+ T cells is termed day 0. Cytokines and fresh medium are added on days 3, 5, and 7. After 10 days of expansion, antigen-reactive CTLs are analyzed.

expansion of antigen-specific CD8+ T cells with an early-differentiated phenotype ( $T_N$  and  $T_{CM}$ ). This observation, though, depended upon on the nature of the antigen because whereas the Melan-A peptide T cells remains with a naïve-like phenotype ( $72.42 \pm 11.53\% T_N$  and  $27.50 \pm 11.51\% T_{CM}$ ), the T cells specific for CMV exhibit mostly a  $T_{CM}$  differentiation phenotype and to a lesser extent a  $T_{EM}$  phenotype ( $15.60 \pm 4.10\% T_N$ ,  $64.25 \pm 13.93\% T_{CM}$  and  $18.90 \pm 8.76\% T_{EM}$ ) (*n* = 5) (Fig. 7a-b). These results were corroborated in a second set of experiments where we compared the effect of IL-2 after a single dose of IL-21 in the final phenotype of the antigen-specific CD8+ T cells expanded. Whereas the combination of IL-21 + IL-7 + 15 promotes the expansion of CD8+ T cells with a predominant  $T_{SCM}$  phenotype, IL-21 + IL-2 induce the differentiation of these cells towards a terminal effector phenotype

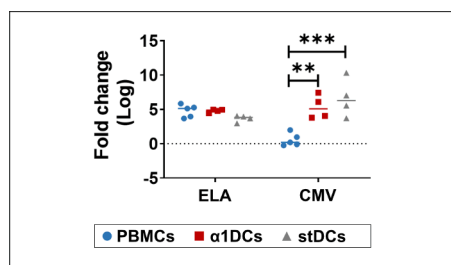
( $T_{EMRA}$ ) (Fig. 7c-d). In contrast, regardless of the antigen source (tumoral or viral), we found that compared to peptide-stimulated PBMCs, the use of moDCs as APCs in media supplemented with IL-21 + IL-7 + 15 promotes the expansion of antigen-specific CD8+ T cells with a predominant  $T_{EM}$  phenotype (whereas  $76.50 \pm 18.26\%$ ,  $80.72 \pm 10.35\%$  and  $76.27 \pm 4.71\%$  of the CD8+ T cells specific for the Melan-A epitope displayed a  $T_{EM}$  phenotype when stimulated with LPS,  $\alpha 1DCs$ , and  $stDCs$  respectively;  $93.02 \pm 5.33\%$  and  $84.81 \pm 8.84\%$  of CMV specific T cells displayed a  $T_{EM}$  phenotype when CD8+ T cells were stimulated with  $\alpha 1DCs$  and  $stDCs$  respectively) (*n* = 4) (Fig. 7e – f. Supplementary Fig S3b-c). Despite  $stDCs$  drive a less vigorous expansion of antigen-specific T cells, we observed that these cells tend to display an early-differentiation state compared to cells expanded in the presence of  $\alpha 1DCs$  (Fig. 7e, ELA panel). Even though this difference was not statistically significant, we decided to study more in depth this observation priming naïve-enriched CD8+ T cells with both  $\alpha 1DCs$  and  $stDCs$  and observed that starting from naïve T cells, the percentage of CMV pp65-specific CD8+ T cells with an early-differentiated phenotype was higher with  $stDCs$  than with  $\alpha 1DCs$  (Supplementary Fig. S4a - b). This observation is intriguing, however, this experiment was carried out on a single donor, so it is required to increase the sample to reach more definitive conclusions.

**3.4.  $\alpha 1DCs$  and  $stDCs$  promote the expansion of non-exhausted Melan-A-specific CD8+ T cells**

A rapid and strong expansion of T cells may jeopardize the integrity of T cells making them susceptible to stimulation-induced cell death. The PD-1, CTLA-4, and KLRG-1 molecules are T cell membrane co-receptors that orchestrate inhibitory signaling pathways impairing the survival and the effector functions of CD8+ T cells. To characterize the senescence/exhaustion phenotype of antigen-specific CD8+ T cells expanded *in-vitro*, we analyzed the expression of PD-1, CTLA-4, and KLRG-1 markers in different cell subpopulations after their expansion with peptide-pulsed- $\alpha 1DCs$  and - $stDCs$ . This analysis revealed that  $T_{EM}$  and  $T_{EMRA}$  subsets of Melan-A/Mart-1<sub>26-35A27L</sub>-specific CD8+ T cells primed in the presence of IL-21 followed by repeated doses of IL-7 and IL-15 in a short 10 days culture exhibited a predominant triple-negative phenotype (PD-1<sup>-</sup>CTLA-4<sup>-</sup>KLRG-1<sup>-</sup>) ( $63.11 \pm 9.34\%$  and  $12.20 \pm 8.80\%$ ;  $T_{EM}$  and  $T_{EMRA}$  respectively), and a PD-1<sup>+</sup>  $T_{EM}$  phenotype ( $17.86 \pm 5.86\%$ ) with no significant difference in the expansion of these populations elicited by the two types of moDCs evaluated (*n* = 4) (Fig. 8).



**Fig. 5. moDCs drive the expansion of antigen-specific CD8+ T cells.** a) Percentage of tetramer-positive CD8+ T cells staining for the Melan-A/Mart-1<sub>26-35A27L</sub> (ELAGIGILTV) and the CMV pp65<sub>495-503</sub> (NLVPMVATV) epitopes after 10 days of *in-vitro* stimulation of enriched-CD8+ T cells with the corresponding peptide-pulsed α1DCs and stDCs; b) Representative dot plots of tetramer-specific CD8+ T cells showed in panel a. The number inside the plots represents the percentage of tetramer-specific CD8+ T cells. Results of experiments presented in panel b are representative of four performed (donors 1–4).



**Fig. 6. PBMCs-based cultures are as efficient as moDCs to expand antigen-specific CD8+ T cells.** Expansion grade of antigen-specific CD8+ T cells from stimulated PBMCs versus moDCs-based cultures. No differences between using moDCs or PBMCs were found for the Melan-A/Mart-1<sub>26-35A27L</sub> epitope. moDCs has a greater capacity expansion than PBMCs-based cultures for the CMV pp65<sub>495-503</sub> epitope.

**4. Discussion**

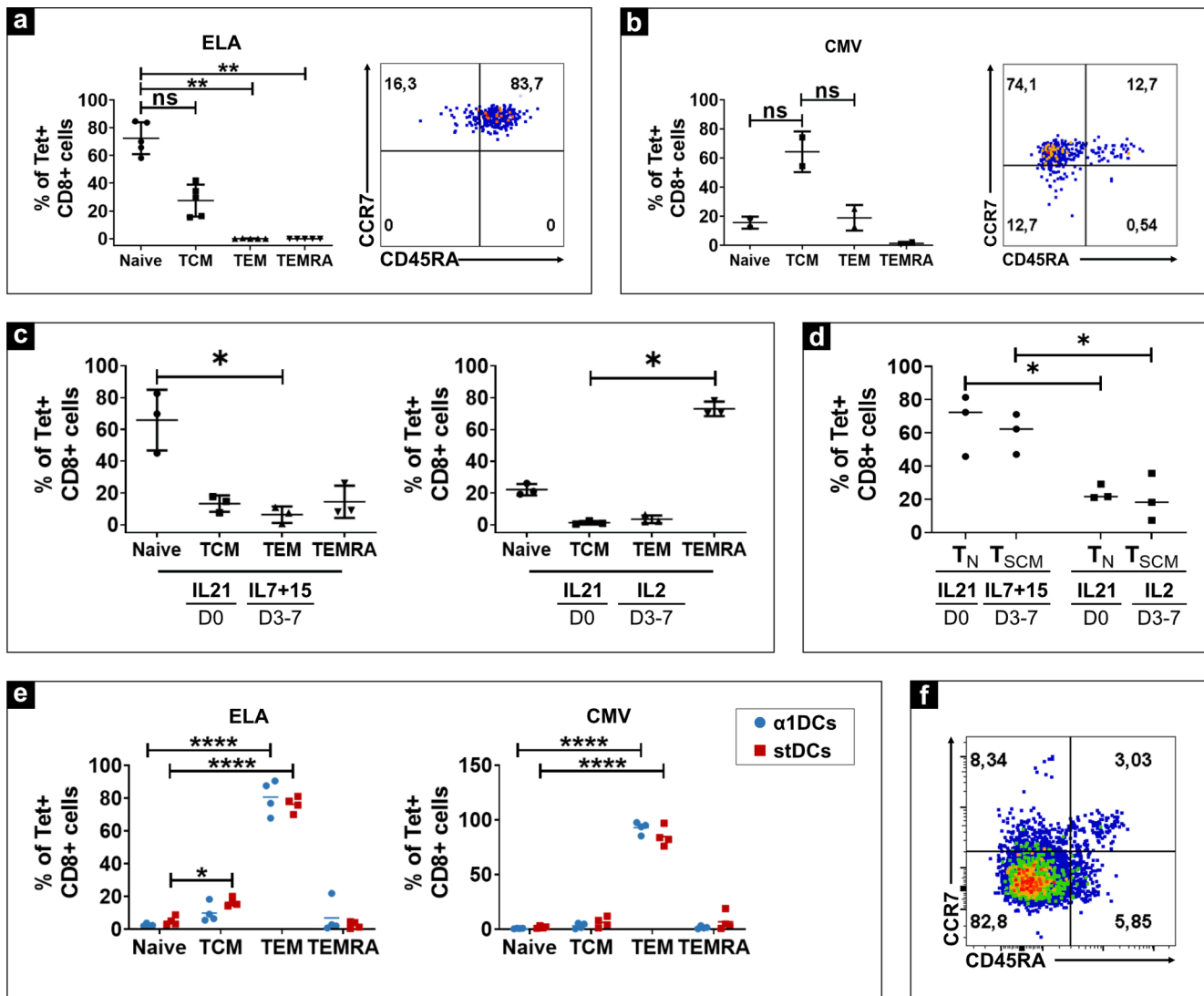
The immune system has the capacity to discriminate between self and non-self-antigens and to eliminate damaged cells and therefore has the potential to eradicate tumor cells that express mutated antigens or self-antigens with aberrant expression [18]. To avoid their elimination by the immune system, tumors not only acquire the ability to be un-recognized but also generate an immunosuppressive tumor microenvironment [19,20]. Therefore, at the end of the 20th century, different strategies of immunotherapy have been explored to reactivate the immune system for the control of cancer [21–23]. One type of widely used immunotherapy is the use of DCs for *in-vitro* or *in-vivo* expansion of antigen-specific CD4+/CD8+ T-cells for the eradication of tumor cells and for the treatment of infectious diseases in immunosuppressed patients [24]. The most used protocol for obtaining DCs is their differentiation from monocytes using GM-CSF and IL-4 followed by their maturation by multiple stimuli [25–27].

Here we evaluated three maturation cocktails: (i) type I alpha cocktail (α1DCs) consisting of IL-6, IL-1β, IFNα, IFNγ, TNFα and a TLR-3-dsRNA synthetic analogue; (ii) standard cocktail (stDCs) containing IL-6, IL-1β, TNFα, and PGE2; and (iii) a cocktail that contains IFNγ and LPS (LPS-DCs). The three types of moDCs obtained reveal morphological and phenotypical changes. On one hand, a transition from a stellate morphology characteristic of immature states to a predominantly veliform one was observed in moDCs (data not shown); as well as the increased expression of CD80, CD83 and CD54. Taking into account these morphological and phenotypic findings, it can be asserted that the four-day protocol implemented to obtain moDCs is comparable to the so-

called two-day fast-DCs and those elicited after the conventional 7-day long culture reported previously [28,29]. However, even though no statistically significant differences in the expression of these markers were found between the moDCs derived with these cocktails, the decrease in the concentration of IL-4 and IFNγ [17], used in our study, seems not to affect neither the maturation process nor the functional capacity of the moDCs compared to those conventional moDCs cultured with these two cytokines with standard concentrations. Considering these findings and the controversial results in previous studies, it is necessary to assess these clinical-grade DCs-maturation cocktails using the same cytokines at lower concentrations and MPLA (Monophosphoryl Lipid A) instead of LPS with the aim of reducing the production costs of dendritic cells for immunotherapy purposes.

In recent years, our group has focused its work on the production of two days derived moDCs for the vaccination of breast cancer patients in neoadjuvant chemotherapy [30,31]. To improve the manufacturing protocols and to extend the range of possibilities of DCs in the field of immunotherapy, here we compare the effectiveness of α1DCs and stDCs versus peptide-stimulated PBMCs to expand antigen-specific CD8+ T cells. According to the literature, α1DCs have a greater capacity to expand antigen-specific T cells than stDCs in total cell numbers [32–39], and the CD8+ T cells expanded by α1DCs perhaps exhibit a cytotoxic phenotype prompted by the high levels of IL-12p70 produced by these DCs. Nevertheless, overall stDCs showed the greatest expansion of CMV pp65 antigen-specific CD8+ T cells (Fig. 5a-b). Although PGE2 added to the proinflammatory cytokine mixture in the standard cocktail accelerates the maturation of DCs increasing the expression of co-stimulatory molecules and their ability to migrate to lymph nodes, it is also known that PGE2 generates an exhausted-DC phenotype compared with DCs obtained with alternative maturation cocktails like α1DCs or LPS-DCs. The exhausted phenotype of stDCs suppresses the production of proinflammatory cytokines critical for T cell activation such as IL-12p70 and increases the release of several suppressive factors such as IL-10 and IDO with the subsequent promotion of Th2 responses [33,40–44]. While the net effect of stDCs is their ability to promote antigen-specific T cell expansion, several clinical and laboratory trials conclude that replacing PGE2 with other factors to drive DC maturation improves the immunogenic and antitumor effectiveness of vaccines developed under this approach [26,45].

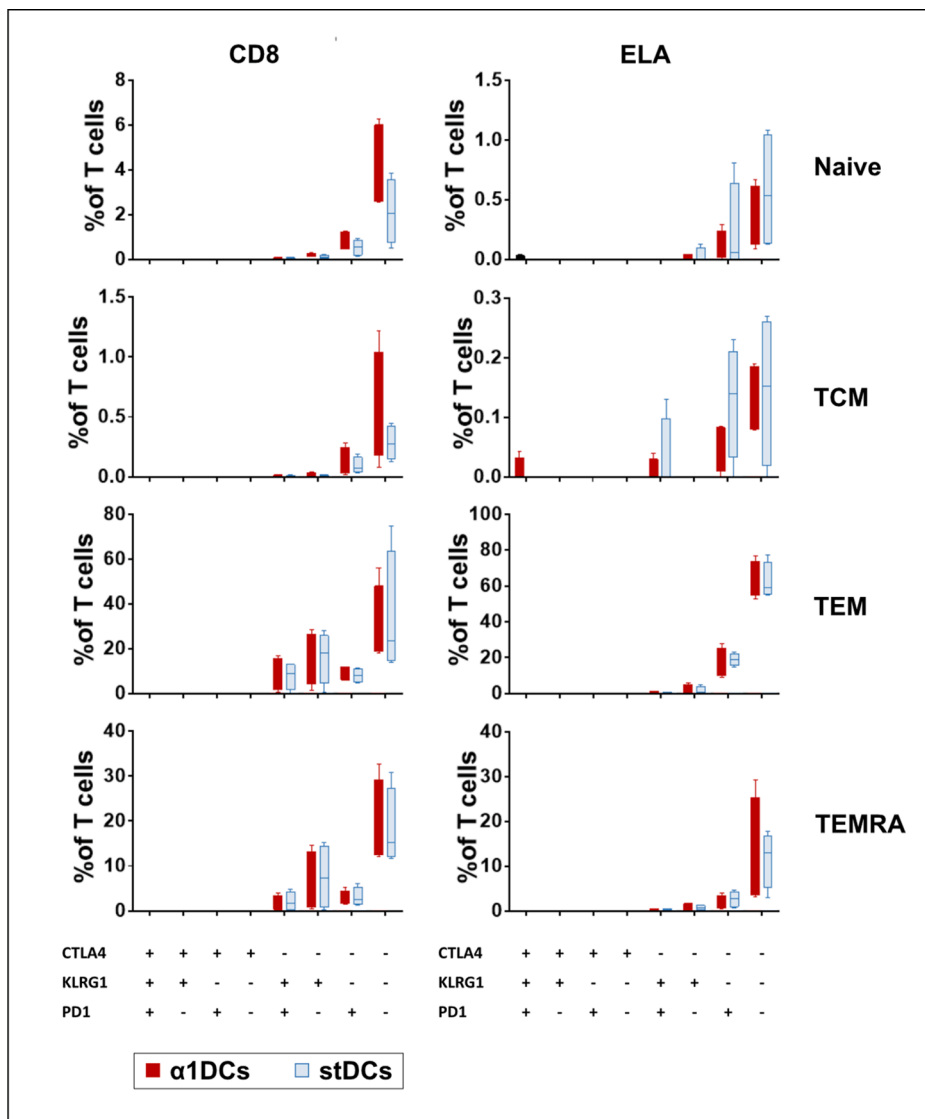
In this work is evident that moDCs induce the expansion of antigen-specific CD8+ T cells either from total CD8+ T cells or naïve CD8+ T cell-enriched fractions, a remarkable benefit to be taken into account for the development of immunotherapy strategies that relies on the *in-vitro* expansion of T cells specific for viral and tumor antigens either from healthy donors or from patient sources. But our group also confirmed the feasibility of expanding antigen-specific CD8+ T cells from peptide-



**Fig. 7.** IL-21, IL-7, and IL-15 drive expansion of antigen-specific CD8<sup>+</sup> T cells with early-differentiated phenotype from peptide-stimulated PBMCs. Expression analysis of CD45RA and CCR7 markers in tetramer-positive CD8<sup>+</sup> T cells after 10 days of Melan-A/Mart-1<sub>26-35A27L</sub> (a) and CMV pp65<sub>495-503</sub> (b) -peptide-stimulated PBMCs (donors 2 – 6). Scatter plots (left panel) and dot plots (right panel) show the percentage of tetramer-positive CD8<sup>+</sup> T cells between the different memory T cells subsets (T<sub>N</sub>, T<sub>CM</sub>, T<sub>EM</sub> and T<sub>EMRA</sub>) of tetramer specific CD8<sup>+</sup> T cells. The number inside the plots represents the percentage of positive or negative markers CD45RA and CCR7 on tetramer-positive CD8<sup>+</sup> T cells. Results of experiments presented in the right panel are representative of five performed; c) Expression analysis of CD45RA and CCR7 markers in tetramer-positive CD8<sup>+</sup> T cells after 10 days of Melan-A/Mart-1<sub>26-35A27L</sub> peptide-stimulated PBMCs (donors 1–3) in the presence of IL-21 + IL-7 + 15 (left panel) or IL-21 + IL-2 (right panel); d) Comparison of the percentage of T<sub>SCM</sub> (CD45RA<sup>+</sup> CCR7<sup>+</sup> CXCR3<sup>+</sup>) subset in tetramer-positive CD8<sup>+</sup> T cells (Melan-A epitope) between two different cytokine conditions; e) Memory markers expression in tetramer-positive CD8<sup>+</sup> T cells after 10 days of enriched-CD8<sup>+</sup> T cells (donors 1 – 4) expansion with α1DCs and stDCs pulsed with Melan-A/Mart-1<sub>26-35A27L</sub> peptide (left panel) or CMV pp65<sub>495-503</sub> peptide (right panel); f) Representative dot plot of memory markers in tetramer specific CD8<sup>+</sup> T cells showed in panel e.

pulsed PBMCs in cytokine conditioned medium using a single dose of IL-21 followed by a combination of IL-7 and IL-15 or with the classical-expansion cytokine IL-2 [46–49]. So, we found differences in the use of moDCs-based cultures or peptide-stimulated PBMCs for the expansion of antigen-specific CD8<sup>+</sup> T cells at least for the two model antigens tested (Melan-A/Mart-1 and CMV pp65). It is not surprising the Melan-A/Mart-1 CD8<sup>+</sup> T cell expansion from PBMCs because the number of naive circulating Melan-A/Mart-1<sub>26-35A27L</sub>-specific CD8<sup>+</sup> T cells from HLA-A2 healthy donors are 100x higher than for any other antigen [50,51]; so it is feasible that the low number of natural DCs and other APCs (like B cells) present in PBMCs are able to expand antigen-specific CD8<sup>+</sup> T cells for the Melan-A/Mart-1 epitope in peptide-pulsed PBMCs cultures. Notably, whereas the use of IL-21 for the first 72 h of the culture of antigen-pulsed PBMCs fosters a vigorous expansion of Melan-A/Mart-1 T cells, it did not promote the expansion of antigen-tested T cells

specific for CMV pp65. This result contrasts with the large expansions of these cells obtained by other researchers using only IL-7 and IL-15 [48]. To confirm our hypothesis that within the PBMCs, IL-21 prime more efficiently naive T cells than memory T cells, it will be necessary to carry out work assessing the priming capacity of IL-21 on naive CD8<sup>+</sup> T cells specific for less frequent antigens such as tumor neoantigens. It is also important to investigate the effect of Th cells in the process of activation and differentiation of the CD8<sup>+</sup> T cells. Several studies have shown that secretion of IL-2 and IL-21 by Th cells and the cell to cell contact with the naive CD8<sup>+</sup> T cells increases the expansion of antigen-specific CD8<sup>+</sup> T cells compared to cultures without the helper stimulus. Although the use of exogenous IL-2 and IL-21 in culture media may favor the expansion of antigen-specific CD8<sup>+</sup> T cells, the absence of CD4<sup>+</sup> T cells slows down the upward regulation of the IL-21 receptor, so that the substitution of this cytokine actually has a limited effect [52]. Moreover, the generation



**Fig. 8. Multiparametric evaluation of the expression profile of inhibitory receptors in antigen-specific CD8+ T cells.** Boolean analysis of the expression of the inhibitory receptors CTLA-4, KLRG-1, and PD-1 on T<sub>N</sub>, T<sub>CM</sub>, T<sub>EM</sub>, and T<sub>EMRA</sub> subsets after 10 days of enriched-CD8+ T cells expansion with α1DCs or stDCs pulsed with Melan-A/Mart-1<sub>26-35A27L</sub> peptide. Box and whisker plot show the percentage of total CD8+ T cells and tetramer-positive CD8+ T cells in each combination marker profile.

and programming of memory in CD8+ T cells relies on helper signals released by CD4 + lymphocytes during CD8 + priming [53]. These signals are transmitted via resident lymph node to cDC1s that are conditioned by CD4 + T cells to release cytokines (IL-12, IL-15) and co-stimulatory signals (CD80, CD86, CD70) that orchestrate the differentiation of naïve CD8 + precursors and their effector capacity [54]. It is important to clarify that these studies have demonstrated that helper signal from CD4 + T cells favors a differentiation program to T<sub>EM</sub> phenotypes with effector capacities on the CD8+ T cells; but also, activates an intrinsic helper gene expression program to face a second antigenic challenge in absence of external helper signals.

In 2011, Gattinoni and colleagues described a new subset of T cells called stem cell-like memory T cells (T<sub>SCM</sub>) identifiable by the presence of both naïve and memory surface markers [9,14]. Replication and renewal capacity of these early-differentiated T cells makes them an excellent candidate to be the basis of long-term immune memory cells suitable for ACTs. Further studies indicate that the use of IL-7, IL-15, and IL-21 (alone or in combination) in the culture media promotes the expansion of this early-differentiated T cells and decreases Tregs subsets generation in contrast to IL-2 [55]. Our results confirmed that the use of IL-21, IL-7, and IL-15 in peptide-stimulated PBMCs cultures promotes the expansion of antigen-specific CD8+ T cells with an early-differentiated phenotype (T<sub>N</sub>, T<sub>SCM</sub> and T<sub>CM</sub>); in contrast to the

effector memory phenotype induced by IL-2. Nevertheless, we did not find the same effect of IL-7 and IL-15 in peptide-pulsed moDCs cultures where the CD8+ T cells expanded have a predominant T<sub>EM</sub> phenotype. This suggests that moDCs may interfere with the ability of common gamma chain cytokines to drive CD8+ T cell expansion with an early-differentiated phenotype. We propose two hypotheses (not mutually exclusive) to explain these findings: (i) the cytokines secreted by moDCs interfere with the ability of IL-21 to retain a naïve phenotype and induce rapid differentiation which cannot be reversed later with the addition of IL-7 and IL-15; and/or (ii) antigenic stimulus mediated by immune synapse between moDCs and T cells through the signaling by MHC-p/TCR complex and costimulatory molecules, are strong and long-lasting signals, that hence, fosters a more differentiated phenotype.

Finally, we evaluated the expression of the inhibitory receptors CTLA-4, PD-1, and KLRG-1. They are essential for central and peripheral tolerance and are responsible for the regulation of the inflammatory response on Melan A/Mart 1<sub>26-35A27L</sub>-specific CD8+ T cells [56]. High expression of these markers in T cells was initially described in the context of chronic viral infections, where antigen-experienced T cells were unable to eliminate infected-host cells by latent viruses such as CMV and EBV; however, several studies have found tumor-infiltrating lymphocyte subsets with this exhausted phenotype. It is believed that under the persistence of the antigen in chronic viral infections and in

growing tumors, mechanisms that regulate the high expression of inhibitory receptors that induces a progressive loss of proliferation and effector functions in T cells are activated. This exhaustion phenomenon is currently considered both a physiological mechanism that limits immunopathology during chronic viral infections but also as one of the major obstacles in the anti-tumor response [57].

CTLA-4 is located in intracellular vesicles and is transiently expressed at the immune synapse after activation by DCs before being rapidly internalized. We are not surprised by the null expression levels of this marker in the T cells expanded concerning these cells received the activation stimulus 10 days before cytometry analysis; furthermore, it should be noted that our analyses are based on CTLA-4 surface staining and not on permeabilized cells, which may be a limitation to be considered in future studies. PD-1 is involved in inhibitory immune signaling and plays a crucial role in the regulation of the adaptive immune response. Most circulating T cells do not express PD-1 but TCR-mediated activation or some cytokines like IL-7, IL-15, and IL-21 (all of which were used in our work) can induce PD-1 expression; furthermore, IFN $\gamma$  (used in  $\alpha$ 1DCs) is known to be the main inducer of PD-L1 (one of the PD-1 ligands) in APCs [58]. Although our research encompasses these two circumstances: the presence of IL-7, IL-15, and IL-21 and the predisposition of  $\alpha$ 1DCs to express higher levels of PD-L1, both CD8 $^+$  T cells and multimer positive CD8 $^+$  T cells showed low levels of this inhibitory receptor.

Unlike PD-1 and CTLA-4, the KLRG-1 co-inhibitory receptor is only expressed on NK and antigen-experienced T cells with late-differentiated phenotypes and not in T<sub>CM</sub> or T<sub>N</sub> subsets [59]. Some studies have suggested that KLRG-1 is a marker of cell senescence because it was found in T cells with poor proliferative capacity after stimulation and in T cells during chronic viral infections and in tumor infiltrating lymphocytes [60,61]. However, a recent study observed that the use of allogeneic DCs induces the expansion of KLRG-1 $^+$  CD8 $^+$  T cells with superior antitumor activity compared to KLRG-1 negative cells, because KLRG-1 $^+$  CD8 $^+$  cells activated by an allogeneic stimulus have a high non-specific antigen effector capacity similar to NK cells [62].

The data obtained allow us to establish that protocols with peptide-pulsed moDCs are as efficient as the peptide-stimulated PBMCs based cultures in the expansion capacity of antigen-specific CD8 $^+$  T cells for the model antigens Melan-A/Mart-1<sub>26-35A27L</sub> and CMV pp65<sub>495-503</sub>. The use of IL-21, IL-7, and IL-15 supplemented media promoted the expansion of antigen-specific CD8 $^+$  T cells with an early-differentiated phenotype (T<sub>SCM</sub> and T<sub>CM</sub>) from peptide-stimulated PBMCs but not when peptide-pulsed moDCs were used as APCs to stimulate enriched CD8 $^+$  T cells. It is possible that the use of naïve cells as starting cells could overcome this obstacle; however, the high number of PBMCs and the high economic costs to obtain naïve-enriched fractions would still be a limitation. We further confirmed that  $\alpha$ 1DCs are superior to stDCs in the activation and expansion of antigen-specific CD8 $^+$  T cells in media supplemented with IL-21, IL-7, and IL-15. These cells displayed a CD45RA $^-$  CCR7 $^-$  CTLA-4 $^-$  PD-1 $^-$  KLRG-1 $^-$  phenotype, that is predominantly antigen experienced effector memory cells with low expression levels of inhibitory co-receptors. Further studies are necessary to clarify the following issues derived from these results: (i) the role of helper signals from CD4 $^+$  T cells in the expansion capacity and memory differentiation process of CD8 $^+$  T cells; (ii) the potential advantage of stDCs over  $\alpha$ 1DCs in the expansion of antigen-specific CD8 $^+$  T cell with T<sub>SCM</sub> phenotype; and (iii) why moDCs promote the expansion of CD8 $^+$  T cells with a T<sub>EM</sub> phenotype instead T<sub>SCM</sub> or T<sub>CM</sub> despite the use of IL-21, IL-7, and IL-15.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Author contribution

Conceived and designed the experiments: JCHM, DBE, CPL.  
 Performed the experiments: JCHM.  
 Analyzed Data: JCHM, DBE, CPL.  
 Contributed reagents/materials/analysis tools: JCHM, DBE, CPL.  
 Wrote the paper: JCHM, DBE, CPL.  
 All authors read and approved the final manuscript.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2020.104257>.

#### References

- [1] S.C. Eisenbarth, Dendritic cell subsets in T cell programming: location dictates function, *Nat. Rev. Immunol.* 19 (2) (2019) 89–103, <https://doi.org/10.1038/s41577-018-0088-1>.
- [2] T.A. Patente, M.P. Pinho, A.A. Oliveira, G.C.M. Evangelista, P.C. Bergami-Santos, J.A.M. Barbutto, Human dendritic cells: Their heterogeneity and clinical application potential in cancer immunotherapy, *Front. Immunol.* 9 (2019) 3176, <https://doi.org/10.3389/fimmu.2018.03176>.
- [3] M. Collin, V. Bigley, Human dendritic cell subsets: an update, *Immunology* 154 (1) (2018) 3–20, <https://doi.org/10.1111/imm.12888>.
- [4] K. Ballen, K. Woo Ahn, M. Chen, H. Abdel-Azim, I. Ahmed, M. Aljurf, J. Antin, A. S. Bhatt, M. Boeckh, G. Chen, C. Dandoy, B. George, M.J. Laughlin, H.M. Lazarus, M.L. MacMillan, D.A. Margolis, D.I. Marks, M. Norkin, J. Rosenthal, A. Saad, B. Savani, H.C. Schouten, J. Storek, P. Szabolcs, C. Ustun, M.R. Verneris, E. K. Waller, D.J. Weisdorf, K.M. Williams, J.R. Wingard, B. Wirk, T. Wolfs, J.-A. Young, J. Auletta, K.V. Komanduri, C. Lindemans, M.L. Riches, Infection Rates among Acute Leukemia Patients Receiving Alternative Donor Hematopoietic Cell Transplantation, *Biol. Blood Marrow Transplantat.* 22 (9) (2016) 1636–1645, <https://doi.org/10.1016/j.bbmt.2016.06.012>.
- [5] R.S. Mehta, K. Rezvani, Immune reconstitution post allogeneic transplant and the impact of immune recovery on the risk of infection, *Virulence* 7 (8) (2016) 901–916, <https://doi.org/10.1080/21505594.2016.1208866>.
- [6] S.A. Rosenberg, N.P. Restifo, J.C. Yang, R.A. Morgan, M.E. Dudley, Adoptive cell transfer: a clinical path to effective cancer immunotherapy, *Nat. Rev. Cancer* 8 (4) (2008) 299–308, <https://doi.org/10.1038/nrc2355>.
- [7] N.P. Restifo, M.E. Dudley, S.A. Rosenberg, Adoptive immunotherapy for cancer: harnessing the T cell response, *Nat. Rev. Immunol.* 12 (4) (2012) 269–281, <https://doi.org/10.1038/nri3191>.

- [8] S.A. Rosenberg, N.P. Restifo, Adoptive cell transfer as personalized immunotherapy for human cancer, *Science* 348 (6230) (2015) 62–68, <https://doi.org/10.1126/science.aaa4967>.
- [9] L. Gattinoni, E. Lugli, Y. Ji, Z. Pos, C.M. Paulos, M.F. Quigley, J.R. Almeida, E. Gostick, Z. Yu, C. Carpenito, E. Wang, D.C. Douek, D.A. Price, C.H. June, F. M. Marincola, M. Roederer, N.P. Restifo, A human memory T cell subset with stem cell-like properties, *Nat. Med.* 17 (10) (2011) 1290–1297, <https://doi.org/10.1038/nm.2446>.
- [10] M.D. Martin, V.P. Badovinac, Defining memory CD8 T cell, *Front. Immunol.* 9 (2018) 2692, <https://doi.org/10.3389/fimmu.2018.02692>.
- [11] L. Gattinoni, D.E. Speiser, M. Lichterfeld, C. Bonini, T memory stem cells in health and disease, *Nat. Med.* 23 (1) (2017) 18–27, <https://doi.org/10.1038/nm.4241>.
- [12] B.-W.L. Xiao-Dong Yuan, Expression Characteristics of Surface Markers of Memory T cells, CD45RO, CCR7 and CD62L, in Tumor-infiltrating Lymphocytes in Liver Cancer Tissues of Patients with Hepatocellular Carcinomas, *J. Clin. Cell. Immunol.* 04 (2013), <https://doi.org/10.4172/2155-9899.1000181>.
- [13] Y.D. Mahnke, T.M. Brodie, F. Sallusto, M. Roederer, E. Lugli, The who's who of T-cell differentiation: Human memory T-cell subsets: HIGHLIGHTS, *Eur. J. Immunol.* 43 (11) (2013) 2797–2809, <https://doi.org/10.1002/eji.201343751>.
- [14] E. Lugli, L. Gattinoni, A. Roberto, D. Mavilio, D.A. Price, N.P. Restifo, M. Roederer, Identification, isolation and in vitro expansion of human and nonhuman primate T stem cell memory cells, *Nat. Protoc.* 8 (1) (2013) 33–42, <https://doi.org/10.1038/nprot.2012.143>.
- [15] J. Lu, R.L. Giuntoli, R. Omiya, H. Kobayashi, R. Kennedy, E. Celis, Interleukin 15 promotes antigen-independent in vitro expansion and long-term survival of antitumor cytotoxic T lymphocytes, *Clin. Cancer Res.* 8 (2002) 3877–3884.
- [16] D.L. Wallace M. Bérard M.V.D. Soares J. Oldham J.E. Cook A.N. Akbar D.F. Tough P.C.L. Beverley Prolonged exposure of naïve CD8 + T cells to interleukin-7 or interleukin-15 stimulates proliferation without differentiation or loss of telomere length : Naïve CD8 T-cell proliferation 119 2 2006 243 253 10.1111/j.1365-2567.2006.02429.x.
- [17] M. Wölfl, P.D. Greenberg, Antigen-specific activation and cytokine-facilitated expansion of naïve, human CD8+ T cells, *Nat. Protoc.* 9 (4) (2014) 950–966, <https://doi.org/10.1038/nprot.2014.064>.
- [18] S. M. Candeias, U. S. Gaip, The immune system in cancer prevention, development and therapy, *Anticancer. Agents Med. Chem.* 16 (1) (2015) 101–107, <https://doi.org/10.2174/18715206150824153523>.
- [19] M. Wang, J. Zhao, L. Zhang, F. Wei, Y.u. Lian, Y. Wu, Z. Gong, S. Zhang, J. Zhou, K. e. Cao, X. Li, W. Xiong, G. Li, Z. Zeng, C. Guo, Role of tumor microenvironment in tumorigenesis, *J. Cancer* 8 (5) (2017) 761–773, <https://doi.org/10.7150/jca.17648>.
- [20] C.G. Drake, E. Jaffee, D.M. Pardoll, Mechanisms of Immune Evasion by Tumors, *Adv. Immunol.* 90 (2006) 51–81, [https://doi.org/10.1016/S0065-2776\(06\)90002-9](https://doi.org/10.1016/S0065-2776(06)90002-9).
- [21] Y. Yang Cancer immunotherapy: harnessing the immune system to battle cancer 125 9 2015 3335 3337 10.1172/JCI83871.
- [22] C. Lee Ventola, Cancer immunotherapy, part 1: Current strategies and agents, *P T.* 42 (2017) 375–383.
- [23] A. Sukari, M. Nagasaka, A. Al-Hadidi, L.G. Lum, Cancer Immunology and Immunotherapy, *AR* 36 (11) (2016) 5593–5606, <https://doi.org/10.21873/anticancer.11144>.
- [24] A. Huber, F. Dammeyer, J.G.J.V. Aerts, H. Vroman, Current State of Dendritic Cell-Based Immunotherapy: Opportunities for in vitro Antigen Loading of Different DC Subsets? *Front. Immunol.* 9 (2018) 2804, <https://doi.org/10.3389/fimmu.2018.02804>.
- [25] M. Saxena, N. Bhardwaj, Re-Emergence of Dendritic Cell Vaccines for Cancer Treatment, *Trends in Cancer* 4 (2) (2018) 119–137, <https://doi.org/10.1016/j.trecan.2017.12.007>.
- [26] K.F. Bol, G. Schreibelt, W.R. Gerritsen, I.J.M. de Vries, C.G. Figdor, Dendritic Cell-Based Immunotherapy: State of the Art and Beyond, *Clin. Cancer Res.* 22 (8) (2016) 1897–1906, <https://doi.org/10.1158/1078-0432.CCR-15-1399>.
- [27] G. Cechim, J.A.B. Chies, In vitro generation of human monocyte-derived dendritic cells methodological aspects in a comprehensive review, *An. Acad. Bras. Cienc.* 91 (2019). <https://doi.org/10.1590/0001-3765201920190310>.
- [28] D.A. Bernal-Estévez, D.T. Tovar Murillo, C.A. Parra- López, Functional and Phenotypic Analysis of Two-Day Monocyte-Derived Dendritic Cells Suitable for Immunotherapy Purposes, *SOJ Immunol.* 4 (2016) 1–18. <https://doi.org/10.15226/2372-0948/4/2/00153>.
- [29] M. Dauer, K. Schad, J. Herten, J. Junkmann, C. Bauer, R. Kiefl, S. Endres, A. Eigler, FastDC derived from human monocytes within 48 h effectively prime tumor antigen-specific cytotoxic T cells, *J. Immunol. Methods* 302 (1-2) (2005) 145–155, <https://doi.org/10.1016/j.jim.2005.05.010>.
- [30] D. Bernal-Estévez, R. Sánchez, R.E. Tejada, C. Parra-López, Chemotherapy and radiation therapy elicits tumor specific T cell responses in a breast cancer patient, *BMC Cancer* 16 (1) (2016), <https://doi.org/10.1186/s12885-016-2625-2>.
- [31] D.A. Bernal-Estévez, O. García, R. Sánchez, C.A. Parra-López, Monitoring the responsiveness of T and antigen presenting cell compartments in breast cancer patients is useful to predict clinical tumor response to neoadjuvant chemotherapy, *BMC Cancer* 18 (1) (2018), <https://doi.org/10.1186/s12885-017-3982-1>.
- [32] R. Trepiakas, A.E. Pedersen, O. Met, M.H. Hansen, A. Bernsten, I.M. Svane, Comparison of  $\alpha$ -Type-1 polarizing and standard dendritic cell cytokine cocktail for maturation of therapeutic monocyte-derived dendritic cell preparations from cancer patients, *Vaccine* 26 (23) (2008) 2824–2832, <https://doi.org/10.1016/j.vaccine.2008.03.054>.
- [33] R.B. Mailliard, A. Wankowicz-Kalinska, Q. Cai, A. Wesa, C.M. Hillkens, M. L. Kapsenberg, J.M. Kirkwood, W.J. Storkus, P. Kalinski,  $\alpha$ -Type-1 Polarized Dendritic Cells: A Novel Immunization Tool with Optimized CTL-inducing Activity, *Cancer Res.* 64 (17) (2004) 5934–5937, <https://doi.org/10.1158/0008-5472.CAN-04-1261>.
- [34] P. Kalinski, H. Okada, Polarized dendritic cells as cancer vaccines: Directing effector-type T cells to tumors, *Semin. Immunol.* 22 (3) (2010) 173–182, <https://doi.org/10.1016/j.smim.2010.03.002>.
- [35] J.-J. Lee R. Mailliard R. Muthuswamy K.A. Foon P. Kalinski Generation of alpha-Type-1 Polarized Dendritic Cells as a Potent Immunogen in Patients with Chronic Lymphocytic Leukemia. 110 11 2007 2059 2059 10.1182/blood. V110.11.2059.2059.
- [36] P. Kalinski, R.B. Mailliard, L. Geskin, A. Giermasz, Y. Nakamura, W.J. Storkus, M. T. Lotze, D.L. Bartlett, J.M. Kirkwood, L.D. Faló, Polarized DC1-Based Therapeutic Cancer Vaccines: *J. Immunother.* 28 (6) (2005) 656, <https://doi.org/10.1097/01.cji.0000191090.97275.78>.
- [37] E. Wieckowski, G.S. Chatta, R.M. Mailliard, W. Gooding, K. Palucka, J. Banchereau, P. Kalinski, Type-1 polarized dendritic cells loaded with apoptotic prostate cancer cells are potent inducers of CD8 + T cells against prostate cancer cells and defined prostate cancer-specific epitopes :  $\alpha$ DC1s Induce Prostate Cancer-specific CTLs, *Prostate* 71 (2) (2011) 125–133, <https://doi.org/10.1002/pros.21228>.
- [38] M.-H. Park, D.-H. Yang, M.-H. Kim, J.-H. Jang, Y.-Y. Jang, Y.-K. Lee, C.-J. Jin, T.N. N. Pham, T.A.N. Thi, M.-S. Lim, H.-J. Lee, C.Y. Hong, J.-H. Yoon, J.-J. Lee, Alpha-type 1 polarized dendritic cells loaded with apoptotic allogeneic breast cancer cells can induce potent cytotoxic T lymphocytes against breast cancer, *Cancer Res Treat* 43 (1) (2011) 56–66, <https://doi.org/10.4143/crt.2011.43.1.56>.
- [39] Y. Akiyama, C. Oshita, A. Kume, A. Iizuka, H. Miyata, M. Komiyama, T. Ashizawa, M. Yagoto, Y. Abe, K. Mitsuya, R. Watanabe, T. Sugino, K. Yamaguchi, Y. Nakasu,  $\alpha$ -type-1 polarized dendritic cell-based vaccination in recurrent high-grade glioma: a phase I clinical trial, *BMC Cancer* 12 (1) (2012), <https://doi.org/10.1186/1471-2407-12-623>.
- [40] P. Kalinski P.L. Vieira J.H.N. Schuitemaker E.C. de Jong M.L. Kapsenberg Prostaglandin E2 is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer 97 11 2001 3466 3469 10.1182/blood.V97.11.3466.
- [41] D.Y. Li, C. Gu, J. Min, Z.H. Chu, Q.J. Ou, Maturation induction of human peripheral blood mononuclear cell-derived dendritic cells, *Exp. Ther. Med.* 4 (2012) 131–134. <https://doi.org/10.3892/etm.2012.565>.
- [42] I. Möller, K. Michel, N. Frech, M. Burger, D. Pfeifer, P. Frommolt, H. Veelken, A.-K. Thomas-Kaskel, Dendritic Cell Maturation With Poly(I:C)-based Versus PGE2-based Cytokine Combinations Results in Differential Functional Characteristics Relevant to the Clinical Application: *J. Immunother.* 31 (5) (2008) 506–519, <https://doi.org/10.1097/CJI.0b013e318177d9e5>.
- [43] S. Trabanelli, M. Lecciso, V. Salvestrini, M. Cavo, D. Očadlíková, R.M. Lemoli, A. Curti, PGE 2-Induced IDO1 Inhibits the Capacity of Fully Mature DCs to Elicit an In Vitro Antileukemic Immune Response, *J. Immunol. Res.* 2015 (2015) 1–10, <https://doi.org/10.1155/2015/253191>.
- [44] W. Jongmans, D.M. Tiemessen, I.J.H. van Vlodrop, P.F.A. Mulders, E. Oosterwijk, Th1-Polarizing Capacity of Clinical-Grade Dendritic Cells Is Triggered by Ribomunyl but Is Compromised by PGE2: The Importance of Maturation Cocktails, *J. Immunother.* 28 (5) (2005) 480–487, <https://doi.org/10.1097/01.cji.0000171290.78495.66>.
- [45] S.K. Wculek, F.J. Cueto, A.M. Mujal, I. Melero, M.F. Krummel, D. Sancho, Dendritic cells in cancer immunology and immunotherapy, *Nat. Rev. Immunol.* 20 (1) (2020) 7–24, <https://doi.org/10.1038/s41577-019-0210-z>.
- [46] B. Eiz-Vesper, B. Maecker-Kolhoff, R. Blasczyk, Adoptive T-cell immunotherapy from third-party donors: Characterization of donors and set up of a T-cell donor registry, *Front. Immunol.* 3 (2013) 410, <https://doi.org/10.3389/fimmu.2012.00410>.
- [47] M. Montes, N. Rufer, V. Appay, S. Reynard, M.J. Pittet, D.E. Speiser, P. Guillaume, J.-C. Cerottini, P. Romero, S. Leyvraz, Optimum in vitro expansion of human antigen-specific CD8+ T cells for adoptive transfer therapy, *Clin. Exp. Immunol.* 142 (2) (2005) 292–302, <https://doi.org/10.1111/j.1365-2249.2005.02914.x>.
- [48] U. Gerdemann, J.M. Keirnan, U.L. Katari, R. Yanagisawa, A.S. Christin, L.E. Huye, S.K. Perna, S. Ennamuri, S. Gottschalk, M.K. Brenner, H.E. Heslop, C.M. Rooney, A. M. Leen, Rapidly Generated Multivirus-specific Cytotoxic T Lymphocytes for the Prophylaxis and Treatment of Viral Infections, *Mol. Ther.* 20 (8) (2012) 1622–1632, <https://doi.org/10.1038/mt.2012.130>.
- [49] U. Gerdemann, U. Katari, A.S. Christin, C.R. Cruz, T. Tripic, A. Rousseau, S. M. Gottschalk, B. Savoldo, J.F. Vera, H.E. Heslop, M.K. Brenner, C.M. Bolland, C. M. Rooney, A.M. Leen, Cytotoxic T Lymphocytes Simultaneously Targeting Multiple Tumor-associated Antigens to Treat EBV Negative Lymphoma, *Mol. Ther.* 19 (12) (2011) 2258–2268, <https://doi.org/10.1038/mt.2011.167>.
- [50] M.J. Pittet D. Valmori P.R. Dunbar D.E. Speiser D. Liénard F. Lejeune K. Fleischhauer V. Cerundolo J.-C. Cerottini P. Romero High Frequencies of Naive Melan-a/Mart-1-Specific Cd8+ T Cells in a Large Proportion of Human Histocompatibility Leukocyte Antigen (Hla)-A2 Individuals 190 5 1999 705 716 10.1084/jem.190.5.705.
- [51] C. Alanio F. Lemaitre H.K.W. Law M. Hasan M.L. Albert Enumeration of human antigen-specific naïve CD8+ T cells reveals conserved precursor frequencies 115 18 2010 3718 3725 10.1182/blood-2009-10-251124.
- [52] M.O. Butler O. Imataki Y. Yamashita M. Tanaka S. Ansén A. Berezovskaya G. Metzler M.I. Milstein M.M. Mooney A.P. Murray H. Mano L.M. Nadler N. Hirano D. Unutmaz Ex Vivo Expansion of Human CD8+ T Cells Using Autologous CD4+ T Cell Help PLoS ONE 7 1 e30229 10.1371/journal.pone.0030229.s003.

- [53] Y.-P. Lai C.-C. Lin W.-J. Liao C.-Y. Tang S.-C. Chen J.H. Fritz CD4+ T Cell-Derived IL-2 Signals during Early Priming Advances Primary CD8+ T Cell Responses PLoS ONE 4 11 e7766 10.1371/journal.pone.0007766.t002.
- [54] T. Ahrends, J. Busselaar, T.M. Severson, N. Bábala, E. de Vries, A. Bovens, L. Wessels, F. van Leeuwen, J. Borst, CD4+ T cell help creates memory CD8+ T cells with innate and help-independent recall capacities, *Nat. Commun.* 10 (2019) 1–13, <https://doi.org/10.1038/s41467-019-13438-1>.
- [55] N. Cieri B. Camisa F. Cocchiarella M. Forcato G. Oliveira E. Provasi A. Bondanza C. Bordignon J. Peccatori F. Ciceri M.T. Lupo-Stanghellini F. Mavilio A. Mondino S. Biciato A. Recchia C. Bonini IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors 121 4 2013 573 584 10.1182/blood-2012-05-431718.
- [56] F.R. Mariotti, L. Quatrini, E. Munari, P. Vacca, L. Moretta, Innate lymphoid cells: Expression of PD-1 and other checkpoints in normal and pathological conditions, *Front. Immunol.* 10 (2019) 910, <https://doi.org/10.3389/fimmu.2019.00910>.
- [57] J.A. Seidel, A. Otsuka, K. Kabashima, Anti-PD-1 and anti-CTLA-4 therapies in cancer: Mechanisms of action, efficacy, and limitations, *Front. Oncol.* 8 (2018) 86, <https://doi.org/10.3389/fonc.2018.00086>.
- [58] E.I. Buchbinder, A. Desai, CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition, *Am. J. Clin. Oncol.* 39 (1) (2016) 98–106, <https://doi.org/10.1097/COC.000000000000239>.
- [59] A. Tata, L. Brossay, Role of the KLRG1 pathway in the immune response, *J. Immunol.* 200 (2018) 49–58.
- [60] L. Li, S. Wan, K. Tao, G. Wang, E. Zhao, KLRG1 restricts memory T cell antitumor immunity, *Oncotarget* 7 (38) (2016) 61670–61678, <https://doi.org/10.18632/oncotarget.11430>.
- [61] S.A. Greenberg, S.W. Kong, E. Thompson, S.V. Gulla, Co-inhibitory T cell receptor KLRG1: human cancer expression and efficacy of neutralization in murine cancer models, *Oncotarget* 10 (14) (2019) 1399–1406, <https://doi.org/10.18632/oncotarget.26659>.
- [62] C. Wang, Z. Li, Z. Zhu, Y. Chai, Y. Wu, Z. Yuan, Z. Chang, Z. Wang, M. Zhang, Allogeneic dendritic cells induce potent antitumor immunity by activating KLRG1 +CD8 T cells, *Sci. Rep.* 9 (1) (2019), <https://doi.org/10.1038/s41598-019-52151-3>.